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ORIGINAL PAPER

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Light effects on cell development and secondary metabolism in *Monascus*

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Abstract In nature, light is one of most crucial environmental signals for developmental and physiological processes in various organisms, including filamentous fungi. We have found that both red light and blue light affect development in *Monascus*, influencing the processes of mycelium and spore formation, and the production of secondary metabolites such as γ-aminobutyric acid, red pigments, monacolin K and citrinin. Additionally, we observed that the wavelength of light affects these developmental and physiological processes in different ways. These findings suggest that *Monascus* possesses a system for differential light response and regulation.

Keywords *Monascus* · Cell development · Secondary metabolism · Red light · Blue light

Introduction

In most organisms light, like temperature, is a crucial environmental signal for regulating developmental and physiological processes. Consequently, the capacity to sense and respond to light is widespread in animals, plants, fungi and bacteria. In *Neurospora crassa*, a well-studied filamentous fungus, it is well known that blue light regulates circadian rhythms and other processes such as the synthesis of carotenoids (photo-protective pigments), spore formation and phototropism of perithecial beaks [12, 13]. The WC-1/WC-2 complex is one of the light input components involved in these processes [12, 13]. WC-1 is a photoreceptor that responds to blue light [8], and also acts as a transcription factor for *frq* (frequency) and other light-regulated genes in conjunction with WC-2 [3, 4, 13]; this complex is modulated by VIVID [15]. The *Neurospora* circadian rhythms are clocked through a light-entrained *frq*-*wc*-based oscillator, dependent on circadian feedback loops [13]. Other light-regulated genes are thought to be controlled by differential WC-1/WC-2 complexes in different ways [13].

Response to other wavelengths of light, such as red light, has been documented in induction of conidiation by red light as well as blue light in *Aspergillus nidulans* [2, 14, 16]. It has been shown that this light dependence is determined by *veA* (velvet) and *fluG* (early acting developmental regulator involved in G-protein signaling) genes in a manner reminiscent of the phytochrome-mediated responses observed in higher plants [16]. Interestingly, both *veA* and *fluG* also affect secondary metabolism, including the synthesis of the polyketide mycotoxin sterigmatocystin, in addition to their roles in developmental processes such as conidiation [10]. Notably, *veA* acts as a global regulator, controlling both secondary metabolism and morphological development, constituting a genetic link between these processes in *A. nidulans* [10]. In addition, *Aspergillus flavus* and *A. nidulans* have recently been shown to have circadian clocks, the components of which have different properties from *wc* and *frq* of *Neurospora* [5].

In fungi other than *Neurospora* some important secondary metabolites are known to be regulated by blue light. Blue light inhibits mycotoxin production in *Aspergillus flavus*, *Aspergillus parasiticus* and *Alternaria alternate* (plant pathogenic fungi) [6].

Monascus species, which have been used in production of traditional oriental foods such as red mold rice, can produce various useful secondary metabolites,

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including red pigments (natural coloring agent), γ-aminobutyric acid (GABA, anti-hypertensive), and monacolin K (MONK, cholesterol-lowering drug) [9]. On the other hand, *Monascus* can also produce the mycotoxin citrinin (a nephrotoxic agent) [1]. GABA is produced simply by decarboxylation of glutamate catalyzed by glutamate decarboxylase, but red pigments, MONK and citrinin are synthesized via the polyketide pathways, which produce many important secondary metabolites with complex chemical structures in filamentous fungi. However, the mechanisms of regulation of these polyketide pathways in *Monascus* have not been elucidated.

Despite the importance of *Monascus* to the food and pharmaceutical industries, light responses in this organism have not been examined. In this study, we examined the effects of constant light exposure on cell development and production of secondary metabolites in *Monascus*.

Materials and methods

Strain and growth conditions

Cultures of *Monascus pilosus*, strain IFO4520 (a citrinin non-producer) and *Monascus purpureus*, strain IFO4478 (a MONK non-producer) were maintained on potato dextrose agar (PDA) (BD, Franklin Lakes, N.J.). After growth on PDA for 10 days at 30°C, spores were harvested with sterile solution (0.9% NaCl, 0.2% Tween 80) and $\sim 10^7$ spores were inoculated in 75 mL potato dextrose (PD) broth (BD) in 200 mL flasks. For MONK and citrinin production, GGP medium (7% glycerol, 3% glucose, 3.8% peptone, 0.1% $MgSO_4 \cdot 7H_2O$, 0.2% $NaNO_3$) was used. Liquid cultures were incubated at 25°C for 14 days at 120 rpm and either kept in the dark or exposed to red light or blue light at 0.16 mW/cm^2. These illuminations, about 635 nm (red) and 470 nm (blue), were produced by red-LED (Stanley, Tokyo, Japan) and blue-LED (Toyoda Gosei, Aichi, Japan), respectively. For growth under anaerobic conditions, liquid cultures were incubated without shaking.

Observation of cell development

In order to observe the effects of light on cell development, PDA plate cultures inoculated with the same number of spores were incubated under dark and light (red or blue) for 10 or 14 days at 30°C. After 10 days of culture, colony mycelium were observed directly by microscope and photographed. After 14 days of culture, spores including asci and conidia were harvested with 10 mL sterile solution (0.9% NaCl, 0.2% Tween 80) and counted using a counting chamber.

Analytical methods

Sample preparation

After liquid culture, fermentation broth and cells were separated by filtration (Advantec No. 5C; Advantec, Tokyo, Japan). Biomass was determined by wet weight of filtered cells from the culture. To prepare cell extracts, 1 g cells (wet weight) was suspended in 60% ethanol, homogenized, and incubated at 25°C for 24 h. Fermentation broth and cell extract were subjected to assay of secondary metabolite production after membrane filtration (pore size, 0.45 μM).

Determination of GABA and red pigments

GABA production was measured by amino acid analysis (Hitachi amino acid analyzer type 835; Hitachi High-Technologies, Tokyo, Japan). Red pigment production was estimated and expressed as absorbance units (U) at 490 nm.

Determination of MONK and citrinin

We established a sensitive and rapid method for both quantitative and qualitative analysis of MONK by HPLC (Agilent 1100 system; Agilent Technologies, Palo Alto, Calif.) connected to TOFMS (JMS-T100LC "AccuTOF"; JEOL, Tokyo, Japan). Prepared samples were analyzed directly using this equipment under the established conditions [HPLC: column, COSMOSIL 5C18-MS (4.6×150 mm; Nakarai Tesque, Kyoto, Japan); linear gradient, acetonitrile-water containing 0.1% formic acid (60:40, v/v) to acetonitrile-water containing 0.1% formic acid (100:0, v/v) in 20 min; flow rate, 0.5 mL/min; oven temperature, 40°C; injection volume, 10 μL. TOFMS: ionization mode, ESI+; needle voltage, 2,000 V; orifice 1 voltage, 70 V; orifice 2 voltage, 5 V; ring lens voltage, 10 V; desolvation chamber temperature, 250°C; analysis time, 20 min; ion detection range, 100–1,000 m/z]. Standards of MONK (acid form, MW = 422; lactone form, MW = 404) in methanol were eluted separately at 10.5–11 and 13.5–14 min and derived to the major ion 445 m/z (M + Na) with minor ion 423 m/z (M + H), and the major ion 427 m/z (M + Na) with 405 m/z (M + H), respectively. In the case of both forms, calibration curves plotted from the total amount of each ion 445/423 and 427/405 m/z were linear from 0.1 to 100 ppm, and the limit of detection was around 0.01 ppm. In this way, the total content of each form of MONK in experimental samples could be determined.

This method could also be used for determination of citrinin levels by chromatograms detected at 260 nm. Citrinin was eluted at 7–7.5 min under these conditions and we have confirmed the presence of citrinin by mass spectra analysis.

Results

Effects of light on cell development

We observed colony formation on PDA plates at 30°C under dark and light (red or blue) exposure. Colony size did not vary under any of the conditions examined. After 10 days of culture, conidiophores observed in mycelia at the tips of colonies under red light were short and the growth was tight compared to colonies grown in the dark (Fig. 1). On the other hand, conidiophores of mycelia grown under blue light were very long and growth was very loose compared to colonies grown in the dark. We also estimated spore formation from each colony under dark and light exposure. Spore formation was stimulated significantly by light exposure (Table 1). However, the number and rate of asci produced increased under red light but not blue light, whereas germination of conidia was stimulated under blue light but not red light. These observations indicate that *M. pilosus* is able to sense and differentiate between light in the red and blue ranges and respond with different patterns of development of mycelium and spore formation.

The biomass of liquid cultures under all conditions was greatest after 10–14 days of culture and no effect of light treatment on biomass was observed (data not shown).

Table 1 Effect of light on spore formation in *Monascus pilosus*. After 14 days of plate culture, colony diameter under dark and light (red and blue) exposure was almost the same (7.5–8 cm)

Light conditions	Number of spores/μL[a]		Rate of asci in spores (%)
	Conidia	Asci	
Dark	643 ± 126	43 ± 15	6.2
Red	1,247 ± 175	120 ± 36	8.7
Blue	1,030 ± 121	50 ± 10	4.6

[a]Spore values are the average of three independent experiments ± SD

Effects of light on GABA production

The GABA content was measured in fermentation broth after incubation in PD broth for 7, 10, and 14 days and in cell extracts at 14 days. Because GABA concentrations were so low in broth culture, we measured GABA in cell extracts. As shown in Fig. 2, blue light apparently increased GABA production compared to cells kept in the dark; red light had no effect under these conditions.

Interestingly, formation of GABA has been shown to be enhanced remarkably under anaerobic conditions [11]. Consequently, we incubated cultures in PD under anaerobic conditions along with parallel batch cultures and analyzed the time course of GABA production in cell extracts. After 14 days of culture, anaerobic cultures kept in the dark showed approximately 2-fold increased GABA production compared to aerated cultures (designated ''dark'' in Figs. 2, 3). Under anaerobic conditions, blue and red light exposure resulted in a 2-fold higher increase in GABA production compared

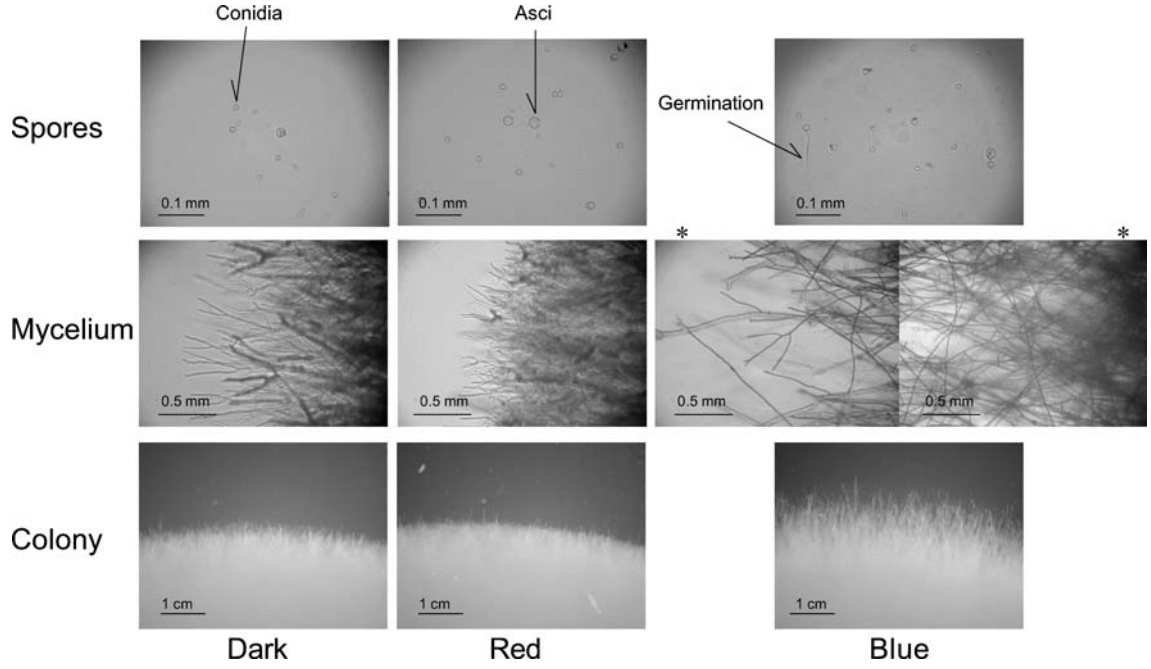

Fig. 1 Effect of light on cell development in *Monascus pilosus*. Plate cultures were kept in the dark, or exposed to red or blue light for 10 or 14 days at 30°C. Formation of mycelium at the tips of colonies was then observed under a microscope, and spores including asci and conidia were harvested, counted, and observed (see Materials and methods). Two panels of mycelium under blue light are shown at the same scale

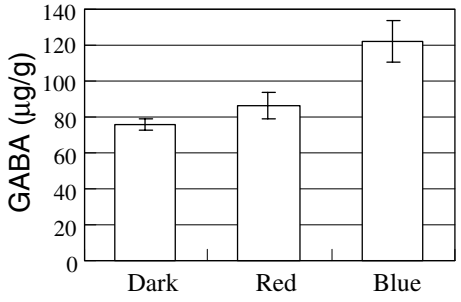

Fig. 2 Effect of light on γ-aminobutyric acid (GABA) production in cell extracts. Cultures of *M. pilosus* IFO4520 in potato dextrose (PD) broth were kept in the dark, exposed to red light or blue light. After 14 days at 25°C, the biomass and GABA content of each cell extract was determined. Values are the average of three independent experiments. *Error bars* SD

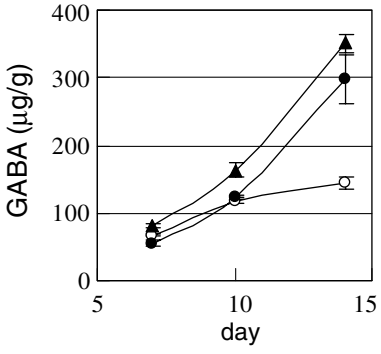

Fig. 3 Time-dependence of GABA production in cell extracts. In parallel, *M. pilosus* IFO4520 cultures in PD broth were kept in the dark (○), or exposed to red (●) or blue (▲) light for 14 days at 25°C. After intervals of 7, 10 and 14 days of culture, the biomass and GABA content of each cell extract was determined. Values are the average of three independent experiments. *Error bars* SD

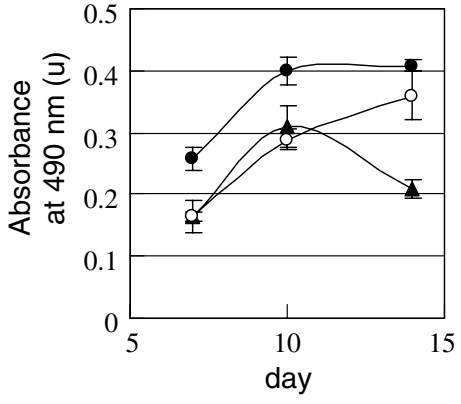

Fig. 4 Time-dependent changes of red pigment production in fermentation broth in response to light. *M. pilosus* IFO4520 cultured in PD broth was kept in the dark (○), or exposed to red (●) or blue (▲) light for 14 days at 25°C. After intervals of 7, 10 and 14 days of culture, red pigment in each fermentation broth was estimated. Values are the average of three independent experiments. *Error bars* SD

to dark conditions (Fig. 3), over that seen in aerated cultures. In addition, under anaerobic conditions, the stimulation of GABA production was observed only after 10 days of incubation (Fig. 3). These results indicate that blue and red light stimulate GABA production in *Monascus*, particularly during the stationary phase of growth.

Effects of light on red pigment production

We also examined the effects of light on red pigment production under the same culture (shaken in PD) and light exposure conditions used for measurement of GABA. Red pigment production was estimated in fermentation broth after 7, 10 and 14 days of culture and also in cell extracts after 14 days of culture. For 7–10 days of culture, red pigment production in fermentation broth increased significantly (about 1.3-fold) upon red light exposure (Fig. 4), although productivity in fermentation broth at 14 days was almost the same. In cell extracts, the stimulating effect (about 1.5-fold) of red light on red pigment production was also observed at 14 days of culture (Fig. 5). These results showed that red light stimulates red pigment production in fermentation broth, especially prior to the stationary phase of growth. Furthermore, this effect seems to be long lasting in the cell.

However, when cultured under blue light, red pigment production estimated in both fermentation broth and cell extract at 14 days of culture was very low compared to production in the dark or under red light exposure. Although this observation suggests an inhibitory effect of blue light on red pigment production, red pigments have a maximum absorbance at 490–500 nm, close to the wavelength of light produced by the blue LED (470 nm). We also observed that blue light appeared to decrease red pigment content in fermentation broth (data not shown). Therefore, whether the observed effect of blue light on red pigment production is a biological process will require further elucidation.

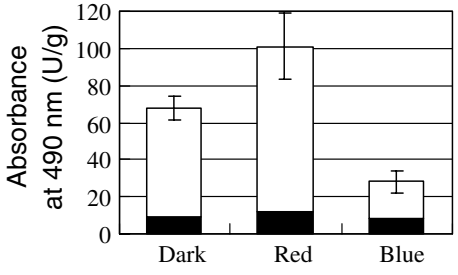

Fig. 5 Effect of light on total red pigment production. Cultures of *M. pilosus* IFO4520 in PD broth were kept in the dark, or exposed to red light or blue light. After 14 days at 25°C, the biomass and red pigment content of each cell extract (*white bars*) and fermentation broth (*black bars*) were estimated. Values are the average of three independent experiments. *Error bars* SD

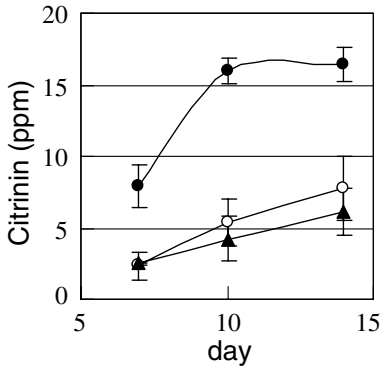

Fig. 6 Time-dependent changes in citrinin production in fermentation broth in response to light. *M. purpureus* IFO4478 cultured in GGP medium was kept in the dark (○), or exposed to red (●) or blue (▲) light for 14 days at 25°C. After intervals of 7, 10 and 14 days of culture, citrinin levels in each fermentation broth were determined. Values are the average of three independent experiments. *Error bars* SD

Effect of light on citrinin production

The mycotoxin citrinin is a derivative of tetraketide, an intermediate in the red pigment synthetic pathway [7]. Using the citrinin-producing strain, *M. purpureus* IFO4478, cultured in GGP medium in the dark or under light (red or blue) exposure, we were able to examine the effects of light on citrinin production in fermentation broth. As shown in Fig. 6, red light significantly stimulated citrinin production during 7–14 days of culture, more strongly during the early stationary phase of growth, and this effect appears to be similar to the effect of red light on red pigment production. On the other hand, blue light had no significant effect.

Effects of light on MONK production

We also examined the red light-dependent production of MONK, another valuable polyketide produced by *M. pilosus* IFO4520. After 7, 10 and 14 days of culture in GGP medium in the dark and under light exposure, the MONK content of *M. pilosus* fermentation broth was determined. The effects of red light exposure on MONK production were similar to those observed for red pigments and citrinin, i.e., at 10 days of culture—corresponding to early stationary phase—MONK production was increased by red light compared to dark (Fig. 7), although MONK production under all conditions reached approximately the same level after 14 days of culture.

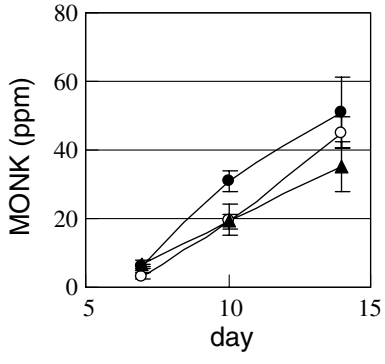

Fig. 7 Time-dependent changes in monacolin K (MONK) production in fermentation broth in response to light. *M. pilosus* IFO4520 cultured in GGP medium was kept in the dark (○), or exposed to red (●) or blue (▲) light for 14 days at 25°C. After intervals of 7, 10 and 14 days of culture, MONK levels in each fermentation broth were determined. Values are the average of three independent experiments. *Error bars* SD

Discussion

Although various developmental processes in *N. crassa* are known to be regulated by blue light, in this study we found that red light also influences cell development, including mycelium and spore formation, in *Monascus*, indicating that light is an important environmental signal for this mold. Red light has also been shown to influence physiological processes in *A. nidulans* [16]. While the responses of mycelium formation to red and blue light differ, both enhance spore formation; however, the point at which the process of spore formation is controlled appears to be different, suggesting a complex relationship between light and development in *Monascus*.

We also found that light influences production of secondary metabolites such as GABA, red pigments, MONK and citrinin in *Monascus*. We observed that red light stimulates production of GABA, red pigments, MONK and citrinin, while blue light stimulates production of GABA, but not the polyketides. It is common for the regulation of GABA production to differ from that of polyketides. Red light was shown to stimulate production of polyketides, particularly early in stationary phase, but production of GABA was effected only at stationary phase, indicating that light regulation of GABA and polyketide production is also different. Taken together, these results suggest that red light affects the regulation of polyketide synthesis rather than its productivity.

We have shown that red light enhances the formation of asci, as well as conidia and short mycelia, while blue light enhances formation of conidia but not asci, and stimulates germination of conidia and mycelial elongation. These observations suggest that red light stimulates the sexual stage of the life cycle of *Monascus*. Any signal that promotes the sexual stage of the life cycle is very important for the operation and/or stimulation of polyketide synthesis in *Monascus*. Furthermore, the stimulation of polyketide synthesis specifically by red light may be attributable to the fact that blue light can degrade red pigments in *Monascus*.

We have shown that *Monascus* responds to light (red and/or blue) by regulating the production of secondary metabolites and cell development. We propose that *Monascus*, *Neurospora*, *Aspergillus* and most likely other fungi as well, possess mechanisms for sensing the quality

of light in a variety of ways. Furthermore, those light-sensing systems may have important commercial applications.

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